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(54) BINDING ASSAY USING BINDING AGENTS WITH TAIL GROUPS

BINDUNGSTEST UNTER VERWENDUNG VON AGENZIEN MIT SCHWANZGRUPPEN

TEST DE FIXATION A L'AIDE D'AGENTS DE FIXATION POSSEDANT DES GROUPES D'EXTREMITE

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Description

Field of the Invention

The present invention relates to binding assays using binding agents with tail groups, and in particular binding agents having oligonucleotide tail groups. These binding assays are useful in determining the concentration of analytes in liquid samples.

Background of the Invention

It is known to measure the concentration of an analyte, such as a drug or hormone, in a liquid sample by contacting the liquid sample with a binding agent immobilised on a solid support, the binding agent having binding sites specific for the analyte, separating the binding agent having analyte bound to it and measuring a value representative of the fraction of the binding sites of the binding agent that are occupied by the analyte. Typically, the concentration of the analyte in the liquid sample can then be determined by comparing the value representative of the fraction of the binding sites occupied by analyte against values obtained from a series of standard solutions containing known concentrations of analyte.

In the past, the measurement of the fraction of the binding sites occupied has usually been carried out by back-titration with a labelled developing reagent using either so-called competitive or non-competitive methods.

In the competitive method, the binding agent having analyte bound to it is back-titrated, either simultaneously or sequentially, with a labelled developing agent, which is typically a labelled version of the analyte or an anti-idiotypic antibody capable of recognising empty binding sites of the binding agent. The developing agent can be said to compete for the binding sites on the binding agent with the analyte whose concentration is being measured.

The fraction of the binding sites which become occupied with the labelled analyte can then be related to the concentration of the analyte as described above.

In the non-competitive method, the binding agent having analyte bound to it is back-titrated with a labelled developing agent capable of binding to either the bound analyte or to the occupied binding sites on the binding agent. The fraction of the binding sites occupied by analyte can then be measured by detecting the presence of the labelled developing agent and, just as with competitive assays, related to the concentration of the analyte in the liquid sample as described above.

In both competitive and non-competitive methods, the developing agent is labelled with a marker to allow the developing agent to be detected. A variety of markers have been used in the past, for example radioactive isotopes, enzymes, chemiluminescent markers and fluorescent markers.

In the field of immunoassay, competitive assays have in general been carried out in accordance with design principles enunciated by Berson and Yalow, for instance in "Methods in Investigative and Diagnostic Endocrinology" (1973), pages 111-116. Berson and Yalow proposed that in the performance of competitive immunoassays, maximum sensitivity is achieved when an amount of binding agent is used to bind approximately 30-50% of a low concentration of the analyte to be detected. In non-competitive immunoassays, maximum sensitivity is generally thought to be achieved by using sufficient binding agent to bind close to 100% of the analyte in the liquid sample. However, in both cases immunoassays designed in accordance with these widely-accepted precepts require the volume of the sample to be known and the amount of binding agent used to be accurately known or known to be constant.

In International Patent Application WO 84/01031, I disclosed that the concentration of an analyte in a liquid sample can be measured by contacting the liquid sample with a small amount of binding agent having binding sites specific for the analyte. In this "ambient analyte" method, provided the amount of binding agent is small enough to have only an insignificant effect on the concentration of the analyte in the liquid sample, it is found that the fraction of the binding sites on the binding agent occupied by the analyte is effectively independent of the volume of the sample.

This approach is further refined in EP 0 304,202 which discloses that the sensitivity and ease of development in the assays in WO 84/01031 are improved by using an amount of binding agent less than 0.1V/K moles located on a small area (or "microspot") on a solid support, where V is the violume of the sample and K is the affinity constant of the binding agent for the analyte. In both of these references, the fraction of the binding sites occupied by the analyte is measured using either a competitive or non-competitive technique as described above.

Summary of the Invention

There is continuing need to develop binding assays which have enhanced kinetics to allow assays to be carried out more quickly and easily. In addition, it would be desirable to provide a binding assay which the user of the assay can easily customise for the detection of different groups of analytes.

Accordingly, in a first aspect, the present invention provides a method of determining the concentrations of analytes

in a liquid sample comprising:

(a) immobilising one or more capture agents on a solid support, each capture agent being capable of specifically binding a given binding agent;
(b) contacting the liquid sample with one or more binding agents, each binding agent having binding sites specific for a given analyte so that a fraction of the binding sites become occupied by the analyte, and a tail group adapted to bind to a corresponding capture agent;
(c) contacting the liquid sample, either simultaneously or sequentially with the step (b), with the immobilised capture agents so that the binding agents become bound to their respective capture agents; and
(d) determining the fraction of the binding sites of a binding agent occupied by analyte to determine the concentration of the analyte in the liquid samples.

Accordingly, the present invention provides an assay in which the binding of the analytes takes place in the liquid phase, rather than at a surface of a solid substrate. This enhances the kinetics of the reaction between analyte and binding agent.

Thus, in one embodiment, contacting the liquid sample with the binding and capture agents simultaneously allows the assay to be carried out in a single step, eg using a single reaction vessel. Alternatively, sequential contact of the binding agent(s) and capture agent(s) may be preferred, especially where the liquid is serum or blood, and nonspecific binding is an important source of error. In these cases, the binding agent can be first contacted with the liquid sample in a first vessel and then the sample transferred to a second vessel to allow the capture agent to bind the binding agent to the solid support.

In a second aspect, the present invention provides a method of immobilising one or more binding agents on a support, each binding agent having binding sites specific for a given analyte and a tail group adapted to bind to a capture agent, comprising:

(a) immobilising one or more capture agents on a support each capture agent being capable of binding to the tail group of a given binding agent and,
(b) contacting the binding agents with the support having the capture agents immobilised thereon so that the binding agents become specifically bound to their respective capture agents through their tail groups.

The above method can additionally comprise the step of attaching the tail groups to the binding agents prior to exposing them to the capture agents immobilised on the support.

Thus, it is possible for the user of the assay to customise binding agents for use in determining the concentration of different groups of analytes and using the customised binding agents in conjunction with a universal support having capture agents immobilised on it, to which the binding agents can individually bind by virtue of their tail groups.

In this aspect of the invention, the assay is carried out by exposing the support to a liquid sample after the binding agent(s) has or have become bound to the capture agent(s).

In either aspect, the present invention provides an assay in which the binding agent is indirectly linked to capture agent immobilised on the substrate via the tail group.

Preferably, the capture agent is an oligonucleotide sequence which can hybridise to a complementary sequence comprising the tail group of the binding agent. The oligonucleotides acting as capture agent or tail of the binding agent are sufficiently long to provide strong and specific hybridisation under the stringency conditions used in the assay. Typically, complementary oligonucleotides of at least about 8 or 9 nucleotides in length are used. In a preferred embodiment, the oligonucleotides are preferably between 8 and 30 bases, more preferably between 16 and 20 bases, in length. However, the use of very long polynucleotides is not preferred as these can lead to a reduction in the specificity of binding different capture agents or to self hybridise, forming hairpin loops (double stranded regions) . However, a suitable length and sequence of oligonucleotide for a set of assay conditions can readily be determined by those skilled in the art.

Conveniently, the binding agent is an antibody having binding sites specific for an analyte. Accordingly, when the capture agent on the support is exposed to the liquid phase binding agent, the binding agent becomes bound to the solid support. Alternatively, where the analyte is a nucleic acid sequence, the binding agent can be an oligonucleotide. Thus, in this embodiment, the binding agent has a first sequence capable of hybridising to the analyte and a second sequence acting as the tail group.

Preferably, a small amount of binding agent is used in accordance with the assays disclosed in WO 84/01031 or EP 0 304,202, so that the volume of the liquid sample need not be known. Thus, the amount of binding agent should be sufficiently small so that it does not significantly affect the ambient concentration of the analyte in the liquid sample. Typically, the use of an amount of binding agent which binds less than 5% of the analyte is preferred. However, the use of a smaller amount of binding agent, eg to bind 2% or 1% of the analyte, further reduces the disturbance to the

ambient concentration of the analyte and helps to minimise the error in determining the analyte concentration.

Where the assay is conducted in accordance with EPO 304,202 using less than 0.1V/K moles of binding agent, the affinity constant (K) for the binding of analyte to binding agent is measured in accordance with normal practice. This means the value of the affinity constant used to determine how much binding agent constitutes 0.1V/K moles is the value that is obtained under the conditions (eg reactants, time of incubation, pH, temperature etc) that are used in the assay.

Preferably, each capture agent is used in excess to bind substantially all of a given binding agent. This maximises the assay sensitivity and ensures that when the amount of binding agent used needs to be known or known to be constant, the user of the assay can be confident that substantially all of a binding agent used in an assay becomes bound to its capture agent on the support.

Preferably, molecules of capture agent are immobilised on a support at discrete locations, eg as microspots. This allows the concentration of a plurality of different analytes to be simultaneously determined using a plurality of different capture agents at a series of locations on the support. Where the capture agent(s) is or are immobilised as microspots, the sensitivity of the assay can be improved immobilising the capture agent at high density, thereby improving the signal-to-noise ratio. Assuming sample volumes of the order of 0.1-1.0 ml, the microspots preferably have an area less than $1mm^2$ and a final surface density of binding agent between 1000 and 100000 molecules/μm^2.

Alternatively, a given capture agent can be immobilised on a support at a plurality of locations so that a series of measurements of the concentration of an analyte can be made simultaneously.

Preferably, the fraction of the binding sites occupied by the analyte is detected using developing agents in a competitive and/or non-competitive method as described above. The developing agents are capable of binding to occupied or unoccupied binding sites of the binding agent or to bound analyte and are labelled to enable bound developing agent to be detected. Preferably, the developing agents are labelled antibodies.

The markers can be radioactive isotopes, enzymes, chemiluminescent markers or fluorescent markers. The use of fluorescent dye markers is especially preferred as the fluorescent dyes can be selected to provide fluorescence of an appropriate colour range (excitation and emission wavelength) for detection. Fluorescent dyes include coumarin, fluorescein, rhodamine and Texas Red. Fluorescent dye molecules having prolonged fluorescent periods can be used, thereby allowing time-resolved fluorescence to be used to measure the strength of the fluorescent signal after background fluorescence has decayed. Latex microspheres containing fluorescent or other markers, or bearing them on their surface can also be employed in this context. The signals from the markers can be measured using a laser scanning confocal microscope.

Alternatively, other high specific activity labels such as chemiluminescent labels can be used. In the case of chemiluminescent labels, the signals from different chemilumiscent labels used to mark binding agent or developing agent can be simultaneously detected using, for example a charge-coupled device (CCD).

The binding agent (or a proportion of it) can conveniently be labelled, eg with a fluorophor. In accordance with the method set out in EPO 271,974, this means that it is not necessary for the user of the assay to know the amount of binding agent or to know that it is constant. This is because the ratio of the signals from the binding agent and the signal indicating the fraction of the binding sites of the binding agent occupied by analyte is dependent on the fraction of the sites of the binding agent occupied by the analyte, but is independent of the total amount of binding agent present.

Alternatively, if the user of the assay knows the volume of the sample, a larger amount of binding agent can be used so that the assay is not operating under ambient analyte conditions. This allows the concentration of the analyte to be determined using one label on the developing agent and either knowing the amount of binding agent is constant or labelling it with a second marker so that the amount is known.

In a variant of this approach, two labelled developing agents can be used, a first capable of specifically binding to unoccupied binding sites of the binding agent and a second capable of binding to occupied binding sites or bound analyte. Thus, the signal from either marker is representative of the fraction of the binding sites occupied by analyte, while the sum of the signals is representative of the total amount of binding agent used.

This method can also avoid the necessity of knowing that a constant amount of binding agent is used as variations in the amount of binding agent immobilised can readily be corrected for. Under these circumstances, the sample volume v must either be known or constant. This can be seen from the following formula show how the signals from two labelled developing agents relates to the concentration of analyte in a sample.

Let the signal emitted by the label marking the developing agent directed against occupied binding agent binding sites be given by S_o,

and the signal emitted by the label marking the developing agent directed against unoccupied binding agent binding sites be given by S_u,
and let the constants relating the respective signals to occupied and unoccupied sites be ε_o and ε_u respectively,
and K = the effective equilibrium constant governing the reaction between the analyte and binding agent.

Then, if the analyte concentration in a sample is given by Y,

$$Y = (S_o/\varepsilon_o)[\varepsilon_u/(KS_u) + 1/v]$$

Assuming v is known, this equation contains two unknown constants, ε_o and ε_u/K. By determining the signals S_o and S_u for a series of known analyte concentrations, these constants can be determined, and unknown analyte concentrations estimated from corresponding determinations of S_o and S_u. Thus, the assay need not work under ambient analyte conditions.

Under ambient analyte conditions, the term $1/v$ becomes negligible, and S_o/S_u is proportional to the ambient analyte concentration.

In a first kit aspect, the present invention provides a kit for determining the concentrations of one or more analytes in a liquid sample in a method as described above, the kit comprising:

(a) a solid substrate having attached thereto at a plurality of locations capture agent capable of specifically binding a binding agent;
(b) one or more binding agents, each binding agent having binding sites specific for an analyte, and a tail group adapted to bind one or more capture agents; and
(c) one or more developing agents having markers capable of binding to occupied binding agent binding sites or analyte bound to binding agent or unoccupied binding agent binding sites.

In a second kit aspect, the present invention provides a kit for customising an assay for the determination of the concentration of one or more analytes comprising:

(a) one or more tail groups, each tail group being for attachment to a binding agent;
(b) a solid substrate having attached thereto at a plurality of locations one or more capture agents capable of specifically binding to a tail group;

wherein the user of the assay attaches the tail groups to the binding agents, thereby providing binding agents which can be used in conjunction with the solid substrate to which the capture agents are attached in a method as described above.

Description of the Drawings

A preferred embodiment of the present invention will now be described with reference to the accompanying schematic drawings in which:

Figure 1 shows an assay to detect two analytes in a liquid sample using two species of capture agent and two species of binding agent, the capture agent immobilised at two microspots;
Figure 2 shows the assay of figure 1 in which the capture agent has become bound to the binding agent;
Figure 3 shows a non-competitive method of determining the occupancy of the binding agent using a second labelled antibody; and,
Figure 4 shows a graph of signal plotted against TSH concentration from the experimental example below.

Detailed Description

Figures 1 to 3 show a binding assay in which two species of binding agent 2,4 having binding sites specific for different analytes 6,8 are used. Each binding agent 2,4 comprises an antibody 10,14 provided with an oligonucleotide tail group 12,16. The oligonucleotide tail groups have different nucleotide sequences, the sequences being complementary to one of the sequences of capture agents 18,20, immobilised on a solid support 22 in the form of microspots. In this example, the oligonucleotides are 8 nucleotides long.

In the assay, the two analytes 6,8 in the sample are exposed to binding agents 2,4 so that a fraction of the analytes 6,8 become bound to the antibodies 10,14. As this reaction occurs in the liquid phase, the kinetics of the reaction between the antibodies 10,14 and the analytes (antigens) 6,8 are optimised.

Simultaneously or sequentially with the initial antibody/analyte reaction, the liquid sample and binding agent are exposed to the solid support 22 having capture agents 18,20 immobilised on it. This allows the nucleotide sequences 12,16 of the binding agents 2,4 to bind to the complementary sequences of the capture agents 18,20 immobilised on the support 22. This is shown in Figure 2. However, the capture agents 16,18 are generally used in excess to ensure

that substantially all the binding agent 10,14 is bound to the support 22. Thus, in figures 2 and 3, one molecule of capture agent 28 is left unoccupied.

The fraction of the binding sites of the binding agents 2,4 can then be determined using a conventional back-titration technique. Thus, in Figure 3 labelled antibodies 24,26 are used in a non-competitive technique to mark the presence of occupied binding agents 2,4 respectively. As the antibodies 24,26 are labelled with markers (not shown) a fraction of the binding sites of the binding agents 2,4 can then be determined. This in turn allows the concentration of the analytes in the liquid sample to be found, eg by reference to results obtained using a series of solutions of known analyte concentration.

The assay shown in Figures 1 to 3 can be adapted to measure the concentration of any pair of analytes using the same solid support 22 having capture agents 18,20 immobilised on it. This can be done by providing binding agent suitable for binding an analyte with an oligonucleotide tail group 12,16 so that the binding agents will specifically bind to one of the microspots 18,20. Thus, it is envisaged that the user of the assay will be able to customise his or her binding agent for use with a universal array of microspots.

Example

Reagents:

1) Mouse IgG (monoclonal anti-TSH) from the Scottish Antibody Production Unit (SAPU).

2) Rabbit IgG, goat anti-mouse IgG (whole molecule) and goat anti-rabbit IgG (whole molecule) antibodies from Sigma.

3) Sulfate Fluospheres, 0.1µm diameter, yellow/green fluorescent (ex 490; em 515nm) and Sulfate Fluospheres, 0.1µm diameter, red fluorescent (ex 580; em 605nm) from Molecular Probes.

4) Oligonucleotides from Oswell DNA Service:

a) CACACACACACACACACA with 5'-biotin modification (poly-CA)

b) GTGTGTGTGTGTGTGTGT with 5'-phosphorothioate modification (poly-GT)

c) GAGAGAGAGAGAGAGAGA with 5'-biotin modification (poly-GA)

d) CTCTCTCTCTCTCTCTCT with 5'-phosphorothioate modification (poly-CT)

5) Sulfo-LC-SPDP {sulfosuccinimidyl 6-[3'-(2-pyridyldithio)-propionamido]hexanoate} from Pierce.

6) PD10 columns and Sephadex G200 from Pharmacia.

7) RIA grade Bovine Serum Albumin (BSA), Tween20, sodium azide, di-sodium hydrogen orthophosphate anhydrous, sodium di-hydrogen orthophosphate, EDTA and Trizma from Sigma

8) Avidin DX from Vector Laboratories

9) Centricon-30 and Centriprep-30 concentrators from Amicon

10) Thyroid stimulating hormone (TSH) from NIH USA

Adsorption of Anti-Mouse IgG and Anti-Rabbit IgG Antibodies to Sulfate FluoSpheres

1) A 0.5ml aliquot of 2% (10mg), 0.1μm yellow/green FluoSpheres was added to 2mg of goat anti-mouse IgG antibody dissolved in 0.5ml 0.1M phosphate buffer, pH7.4. A 0.5ml aliquot of 2% (10mg), 0.lum red FluoSpheres was added to 2mg of goat anti-rabbit IgG antibody dissolved in 0.5ml 0.1M phosphate buffer, pH7.4. Both preparations were shaken overnight at room temperature.

2) The two preparations were centrifuged for 10min at 8°C in a MSE High-Spin 21 Ultra-centrifuge.

3) Each pellet was dispersed in 2ml of 1% BSA in phosphate buffer, shaken for 1 hour at room temperature and centrifuged as above.

4) Each pellet was dispersed in 2ml of 0.5% Tween20 in phosphate buffer, shaken for 30min at room temperature and centrifuged as above.

5) Each pellet was dispersed in 2ml of phosphate buffer and centrifuged as above.

6) Each pellet was dispersed in 2ml of phosphate buffer and centrifuged as above.

7) Each pellet was dispersed in 2ml of 1% BSA containing 0.1% sodium azide and stored at 4°C.

Conjugation of Mouse Monoclonal IgG and Rabbit IgG to Oligonucleotides

1) 3mg of sulpho-LC-SPDP was added to 4.6mg of mouse anti-TSH monoclonal or rabbit IgG dissolved in Iml of PBS/EDTA and shaken for 30min at room temperature.

2) The activated antibodies were separated from unreacted SPDP on PD10 columns. The samples were eluted with PBS/EDTA and 0.5ml fractions collected.

3) The fractions from the first peak containing the activated antibody were pooled and concentrated using a Centricon-30 concentrator to approximately 10μl.

4) 100nM of 5'-phosphorothioate modified poly-GT oligonucleotide was added to 14.8nM of the activated mouse monoclonal IgG. 58.3nM of 5'-phosphorothioate modified poly-CT oligonucleotide was added to 8.7nM of the activated rabbit IgG. Both preparations were made up to Iml with PBS/EDTA and shaken overnight at room temperature.

5) The oligonucleotide conjugated mouse and rabbit IgG preparations were separated from unreacted oligonucleotides on a Sephadex G200 column (1.5 x 45cm). The samples were eluted with PBS/EDTA and 2ml fractions collected.

6) The fractions from the first peak containing the oligonucleotide conjugated antibody were pooled and concentrated using a Centriprep-30 concentrator to approximately 500μl and stored at 4°C.

To Demonstrate That a Mixture of Oligonucleotide-Conjugated Antibodies Would Hybridize Only With Complementary Oligonucleotide Deposited on a Solid-Phase as Microspots

1) Dynatech black Microfluor microtitre wells were coated with 50μl of avidin-DX in 0.1M bicarbonate buffer, pH 8.5 and at a concentration of 5μg/ml for 5min at room temperature.

2) After washing with 0.01M phosphate buffer, the avidin coated microtitre wells were blocked with 200μl of 1% BSA for 1 hour at room temperature and washed again with the same buffer and dried.

3) A 0.25µl droplet of each of the two 5'-biotin modified poly-CA and poly-GA oligonucleotides in 0.1% BSA and at a concentration of 0.025nM/ml were deposited on opposite sides of avidin coated microtitre wells and allowed to react for 30min under a moist atmosphere. The droplets were then aspirated and the microtitre wells washed with phosphate buffer.

4) A 50µl aliquot of Tris-HCl assay buffer containing 0.25ug/ml each of the poly-GT-conjugated mouse monoclonal IgG and poly-CT-conjugated rabbit IgG was added to all but the control microtitre wells (50µl of assay buffer containing unconjugated mouse and rabbit IgG was added to the control wells instead), shaken for 1 hour under a moist atmosphere and washed with phosphate buffer containing 0.05% Tween20.

5) A 200µl aliquot of Tris-HCl assay buffer containing 0.3µg/ml goat anti-mouse IgG antibody conjugated yellow/green FluoSpheres and 0.6µg/ml goat anti-rabbit IgG antibody conjugated red FluoSpheres was added to all microtitre wells, shaken for 1 hour at room temperature, washed with phosphate-Tween20 buffer and scanned with a confocal laser scanning microscope equipped with an Argon/Krypton laser.

<u>**Results**</u>

Excitation: 488DF10
Emission: 525DF35

Sample	Yellow/Green Signal
Control	13.3±0.5
Avidin--B-Poly-CA---Poly-GT-Mouse IgG microspot	100.9±10.9
Avidin--B-Poly-GA---Poly-CT-Rabbit IgG microspot	16.9±0.3

Excitation: 568DF10
Emission: 585EFLP

Sample	Red Signal
Control	22.0±0.2
Avidin---B-Poly-CA---Poly-GT-Mouse IgG microspot	24.0±0.4
Avidin---B-Poly-GA---Poly-CT-Rabbit IgG microspot	99.8±2.7

<u>**Conclusions**</u>

(1) The poly-GT oligonucleotide tagged mouse IgG hybridized only with complementary biotinylated poly-CA but not non-complementary biotinylated poly-GA oligonucleotide microspots deposited on the same microtitre well.

(2) The poly-CT oligonucleotide tagged rabbit IgG hybridized only with complementary biotinylated poly-GA but not non-complementary biotinylated poly-CA oligonucleotide microspots deposited on the same microtitre well.

To Demonstrate Antigen Binding of the Oligonucleotide Tagged Antibody Microspots

1) Dynatech black Microfluor microtitre wells were coated with 50µl of avidin-DX in 0.1M bicarbonate buffer, pH 8.5 and at a concentration of 5ug/ml for 5min at room temperature.

2) After washing with 0.01M phosphate buffer, the avidin coated microtitre wells were blocked with 200µl of 1% BSA for 1 hour at room temperature and washed again with the same buffer and dried..

3) A 0.25 droplet of 5'-biotin modified poly-CA oligonucleotide in 0.1% BSA and at a concentration of 0.025nM/ml was deposited on each of the avidin coated microtitre wells and allowed to react for 30min under a moist atmos-

phere. The droplets were then aspirated and the microtitre wells washed with phosphate buffer.

4) A 50µl aliquot of Tris-HCl assay buffer containing 0.25µg/ml of the poly-GT-conjugated anti-TSH mouse monoclonal IgG was added to the microtitre wells, shaken for 1 hour under a moist atmosphere and washed with phosphate buffer containing 0.05% Tween20.

5) A 200µl aliquot of TSH standards in Tris-HCl assay buffer (0, 0.1, 0.3 & 1.0µU/ml) was added to triplicate wells and incubated for 1 hour at room temperature and washed with phosphate-Tween20 buffer.

6) A 200µl aliquot of 50µg/ml anti-TSH developing antibody conjugated yellow/green sulfate FluoSpheres was added to all microtitre wells, shaken for 1 hour at room temperature, washed with phosphate-Tween20 buffer and scanned with a confocal laser scanning microscope equipped with an Argon/Krypton laser.

Results and Conclusion

The poly-GT oligonucleotide tagged anti-TSH mouse monoclonal IgG was fully functional as demonstrated by the successful generation of a standard curve when it was used as binding antibody deposited on the solid-phase via biotinylated complementary poly-CA oligonucleotide coupled to avidin coated microtitre wells (see figure 4).

Claims

1. A method for determining the concentration of one or more analytes in a liquid sample comprising:

   (a) immobilising one or more capture agents on a solid support, each capture agent being capable of specifically binding a given binding agent;
   (b) contacting the liquid sample with one or more binding agents, each binding agent having binding sites specific for a given analyte so that a fraction of the binding sites become occupied by the analyte, and having a tail group adapted to bind to the corresponding capture agent;
   (c) contacting the liquid sample, either simultaneously or sequentially with the step (b), with the immobilised capture agents so that the binding agents become bound to their respective capture agents; and,
   (d) determining a value representative of the fraction of the binding sites of a given binding agent occupied by an analyte whereby to determine the concentration of the analyte in the liquid sample

   wherein the capture agents are oligonucleotides having sequences which can hybridise to a complementary sequence on the tail group of the corresponding binding agent.

2. A method according to claim 1 wherein the method additionally comprises the step of attaching the tail groups to the binding agents prior to immobilising them on the support.

3. A method according to claim 1 or claim 2 wherein the oligonucleotides are between 8 and 30 bases long.

4. A method according to any one of the preceding claims wherein the binding agent is an antibody having binding sites specific for an analyte.

5. A method according to any one of the preceding claims wherein a small amount of each binding agent is used so that the ambient concentration of the analyte for which the binding agent is specific is not significantly disturbed.

6. A method according to claim 5 wherein the small amount of binding agent is less than 0.1V/K moles, where V is the volume of the sample and K is the effective affinity constant for the analyte binding to the binding agent.

7. A method according to any one of the preceding claims wherein each capture agent is used in excess to bind substantially all of a given binding agent.

8. A method according to any one of the preceding claims wherein the capture agent(s) are immobilised on a support at discrete locations.

9. A method according to claim 8 wherein the discrete locations are microspots.

10. A method according to any one of the preceding claims wherein a given capture agent is immobilised on the support at a plurality of locations so that a series of measurements of the concentration of a given analyte can be made simultaneously.

11. A method according to any one of the preceding claims wherein the value representative of the fraction of the binding sites occupied by the analyte is determined using developing agents in a competitive and/or non-competitive method, the developing agents being labelled with markers.

12. A method according to claim 11 wherein the marker are fluorescent or chemiluminescent markers.

13. A kit for determining the concentrations of one or more analytes in a liquid sample in a method according to any one of claims 1 to 12, the kit comprising:

    (a) a solid substrate having attached thereto at a plurality of locations capture agent capable of specifically binding a given binding agent;
    (b) one or more binding agents, each binding agent having binding sites specific for an analyte, and having a tail group adapted to bind one or more capture agents; and
    (c) one or more developing agents having markers capable of binding to occupied binding agent binding sites or analyte bound to binding agent or unoccupied binding agent binding sites;

    wherein the capture agents are oligonucleotides having sequences which can hybridise to a complementary sequence on the tail group of the corresponding binding agent.

14. A kit for customising an assay for the determination of the concentration of one or more analytes in a liquid sample, the kit comprising:

    (a) one or more tail groups, each tail group being for attachment to a binding agent;
    (b) a solid substrate having attached thereto at a plurality of locations one or more capture agents capable of specifically binding to a tail group;

    wherein the capture agents are oligonucleotides having sequences which can hybridise to a complementary sequence on the tail group of the corresponding binding agent and the user of the assay attaches the tail groups to the binding agents, thereby providing binding agents which can be used in conjunction with the solid substrate to which the capture agents are attached in a method according to any one of claims 1 to 12.

Patentansprüche

1. Verfahren zum Bestimmen der Konzentration eines oder mehrerer Analyten in einer flüssigen Probe, umfassend:

    (a) das Immobilisieren eines oder mehrerer Fänger auf einem festen Träger, wobei jeder Fänger zum spezifischen Binden eines bestimmten Bindemittels fähig ist;
    (b) das In-Kontakt-Bringen der flüssigen Probe mit einem oder mehreren Bindemitteln, wobei jedes Bindemittel Bindungsstellen, die für einen bestimmten Analyten spezifisch sind, so daß ein Teil der Bindungsstellen vom Analyten besetzt wird, sowie eine Schwanzgruppe aufweist, die so ausgebildet ist, daß sie sich an den entsprechenden Fänger bindet;
    (c) das In-Kontakt-Bringen der flüssigen Probe mit den immobilisierten Fängern, wodurch die Bindemittel an ihre jeweiligen Fänger gebunden werden, entweder gleichzeitig mit oder nach Schritt (b), und
    (d) das Bestimmen eines Werts, der für den Anteil der Bindungsstellen eines bestimmten Bindemittels, die von einem Analyten besetzt sind, repräsentativ ist, um dadurch die Konzentration des Analyten in der flüssigen Probe zu bestimmen,

    wobei die Fänger Oligonukleotide sind, die Sequenzen aufweisen, die an eine komplementäre Sequenz der Schwanzgruppe des entsprechenden Bindemittels hybridisieren können.

2. Verfahren nach Anspruch 1, worin das Verfahren zusätzlich den Schritt des Bindens der Schwanzgruppe an die Bindemittel vor dem Immobilisieren derselben auf dem Träger umfaßt.

3. Verfahren nach Anspruch 1 oder 2, worin die Oligonukleotide zwischen 8 und 30 Basen lang sind.

4. Verfahren nach einem der vorangegangenen Ansprüche, worin das Bindemittel ein Antikörper ist, der für einen Analyten spezifische Bindungsstellen aufweist.

5. Verfahren nach einem der vorangegangenen Ansprüche, worin eine geringe Menge jedes Bindemittels so eingesetzt wird, daß die Umgebungskonzentration des Analyten, für den das Bindemittel spezifisch ist, nicht nennenswert gestört wird.

6. Verfahren nach Anspruch 5, worin die geringe Menge an Bindemittel weniger als 0,1 V/K Mol beträgt, worin V das Volumen der Probe ist und K die wirksame Affinitätskonstante für den Analyten ist, der sich an das Bindemittel bindet.

7. Verfahren nach einem der vorangegangenen Ansprüche, worin jeder Fänger im Überschuß eingesetzt wird, um im wesentlichen jeweils das gesamte Bindemittel zu binden.

8. Verfahren nach einem der vorangegangenen Ansprüche, worin der/die Fänger auf einem Träger an diskreten Positionen immobilisiert wird/werden.

9. Verfahren nach Anspruch 8, worin die diskreten Positionen Mikropunkte sind.

10. Verfahren nach einem der vorangegangenen Ansprüche, worin ein bestimmter Fänger auf einem Träger an einer Vielzahl von Positionen immobilisiert wird, so daß gleichzeitig eine Reihe von Messungen der Konzentration eines bestimmten Analyten durchgeführt werden kann.

11. Verfahren nach einem der vorangegangenen Ansprüche, worin der Wert, der für den Anteil der Bindungsstellen repräsentativ ist, die vom Analyten besetzt werden, unter Verwendung von Entwicklern in einem kompetitiven und/oder nicht-kompetitiven Verfahren ermittelt wird, wobei die Entwickler mit Markierungen markiert sind.

12. Verfahren nach Anspruch 11, worin die Markierungen Fluoreszenz- oder Chemolumineszenz-Markierungen sind.

13. Set zum Bestimmen der Konzentrationen eines oder mehrerer Analyten in einer flüssigen Probe mittels eines Verfahrens nach einem der Ansprüche 1 bis 12, wobei das Set umfaßt:

(a) ein festes Substrat, an dem an einer Vielzahl von Positionen Fänger gebunden sind, die dazu fähig sind, ein bestimmtes Bindemittel spezifisch zu binden;
(b) ein oder mehrere Bindemittel, wobei jedes Bindemittel Bindungsstellen, die für einen Analyten spezifisch sind, sowie eine Schwanzgruppe aufweist, die zum Binden eines oder mehrerer Fänger ausgebildet ist; und
(c) einen oder mehrere Entwickler, die Markierungen aufweisen, die zur Bindung an besetzte Bindemittel-Bindungsstellen oder an an Bindemittel gebundenen Analyten oder an unbesetzte Bindemittel-Bindungsstellen fähig sind;

wobei die Fänger Oligonukleotide sind, die Sequenzen aufweisen, die an eine komplementäre Sequenz an der Schwanzgruppe des entsprechenden Bindemittels hybridisieren können.

14. Set zur Individualisierung eines Assays zur Bestimmung der Konzentration eines oder mehrerer Analyten in einer flüssigen Probe, wobei das Set umfaßt:

(a) eine oder mehrere Schwanzgruppen, wobei jede Schwanzgruppe zur Bindung an ein Bindemittel bestimmt ist;
(b) ein festes Substrat, an dem an einer Vielzahl von Positionen ein oder mehrere Fänger befestigt ist/sind, der/die zur spezifischen Bindung an eine Schwanzgruppe fähig ist/sind;

wobei die Fänger Oligonukleotide sind, die Sequenzen aufweisen, die an eine komplementäre Sequenz der Schwanzgruppe des entsprechenden Bindemittels hybridisieren können, und der Benutzer des Assays die Schwanzgruppen an die Bindemittel bindet, wodurch Bindemittel bereitgestellt werden, die in Verbindung mit dem festen Substrat, an dem die Fänger befestigt sind, für ein Verfahren nach einem der Ansprüche 1 bis 12 eingesetzt werden können.

Revendications

1. Méthode de détermination de la concentration d'un ou plusieurs analytes dans un échantillon liquide comprenant:

   (a) l'immobilisation d'un ou plusieurs agents de capture sur un support solide, chaque agent de capture étant capable de lier spécifiquement un agent de liaison donné ;
   (b) la mise en contact de l'échantillon liquide avec un ou plusieurs agents de liaison, chaque agent de liaison ayant des sites de liaison spécifiques pour un analyte donné de sorte qu'une fraction des sites de liaison devient occupée par l'analyte et ayant un groupe de queue adapté à la liaison à l'agent de capture correspondant ;
   (c) la mise en contact de l'échantillon liquide, soit simultanément soit en séquence avec l'étape (b), avec les agents de capture immobilisés de sorte que les agents de liaison deviennent liés à leurs agents de capture respectifs ; et
   (d) la détermination d'une valeur représentative de la fraction des sites de liaison d'un agent de liaison donné occupée par un analyte pour déterminer la concentration de l'analyte dans l'échantillon liquide

   dans laquelle les agents de capture sont des oligonucléotides ayant des séquences qui peuvent s'hybrider à une séquence complémentaire sur le groupe de queue de l'agent de liaison correspondant.

2. Méthode selon la revendication 1, dans laquelle la méthode comprend en outre l'étape de fixer les groupes de queue aux agents de liaison avant de les immobiliser sur le support.

3. Méthode selon la revendication 1 ou 2, dans laquelle les oligonucléotides ont une longueur d'entre 8 et 30 bases.

4. Méthode selon l'une quelconque des revendications précédentes, dans laquelle l'agent de liaison est un anticorps ayant des sites de liaison spécifiques pour un analyte.

5. Méthode selon l'une quelconque des revendications précédentes, dans laquelle une petite quantité de chaque agent de liaison est utilisée de sorte que la concentration ambiante de l'analyte pour lequel l'agent de liaison est spécifique ne soit pas perturbée de façon significative.

6. Méthode selon la revendication 5, dans laquelle la petite quantité d'agent de liaison est inférieure à 0,1 V/K moles, V étant le volume de l'échantillon et K est la constante d'affinité effective pour la liaison de l'analyte à l'agent de liaison.

7. Méthode selon l'une quelconque des revendications précédentes, dans laquelle chaque agent de capture est utilisé en excès pour lier sensiblement la totalité d'un agent de liaison donné.

8. Méthode selon l'une quelconque des revendications précédentes, dans laquelle l'agent (les agents) de capture sont immobilisés sur un support en des endroits séparés.

9. Méthode selon la revendication 8, dans laquelle les endroits séparés sont des micropositions.

10. Méthode selon l'une quelconque des revendications précédentes, dans laquelle un agent de capture donné est immobilisé sur le support en une pluralité d'endroits de sorte qu'on puisse effectuer simultanément une série de mesures de la concentration d'un analyte donné.

11. Méthode selon l'une quelconque des revendications précédentes, dans laquelle la valeur représentative de la fraction des sites de liaison occupée par l'analyte est déterminée en utilisant des agents de développement dans une méthode compétitive et/ou non compétitive, les agents de développement étant marqués avec des marqueurs.

12. Méthode selon la revendication 11, dans laquelle les marqueurs sont des marqueurs fluorescents ou chimioluminescents.

13. Trousse de détermination des concentrations d'un ou plusieurs analytes dans un échantillon liquide dans une méthode selon l'une quelconque des revendications 1 à 12, la trousse comprenant :

    (a) un substrat solide ayant fixé en une pluralité d'endroits un agent de capture capable de lier spécifiquement

un agent de liaison donné;
(b) un ou plusieurs agents de liaison, chaque agent de liaison ayant des sites de liaison spécifiques pour un analyte et ayant un groupe de queue adapté à la liaison d'un ou plusieurs agents de capture ; et
(c) un ou plusieurs agents de développement ayant des marqueurs capables de se lier aux sites de liaison de l'agent de liaison occupés ou à un analyte lié à l'agent de liaison ou aux sites de liaison de l'agent de liaison non occupés,

dans laquelle les agents de capture sont des oligonucléotides ayant des séquences qui peuvent s'hybrider à une séquence complémentaire sur le groupe de queue de l'agent de liaison correspondant.

14. Trousse pour adapter un essai pour la détermination de la concentration d'un ou plusieurs analytes dans un échantillon liquide, la trousse comprenant :

(a) un ou plusieurs groupes de queue, chaque groupe de queue existant pour s'attacher à un agent de liaison ;
(b) un substrat solide ayant, attachés en une pluralité d'endroits, un ou plusieurs agents de capture capables de se lier spécifiquement à un groupe de queue ;

dans laquelle les agents de capture sont des oligonucléotides ayant des séquences qui peuvent s'hybrider à une séquence complémentaire sur le groupe de queue de l'agent de liaison correspondant et l'utilisateur de l'essai attache les groupes de queue aux agents de liaison, fournissant ainsi des agents de liaison qui peuvent être utilisés en combinaison avec le substrat solide auquel les agents de capture sont fixés dans une méthode selon l'une quelconque des revendications 1 à 12.

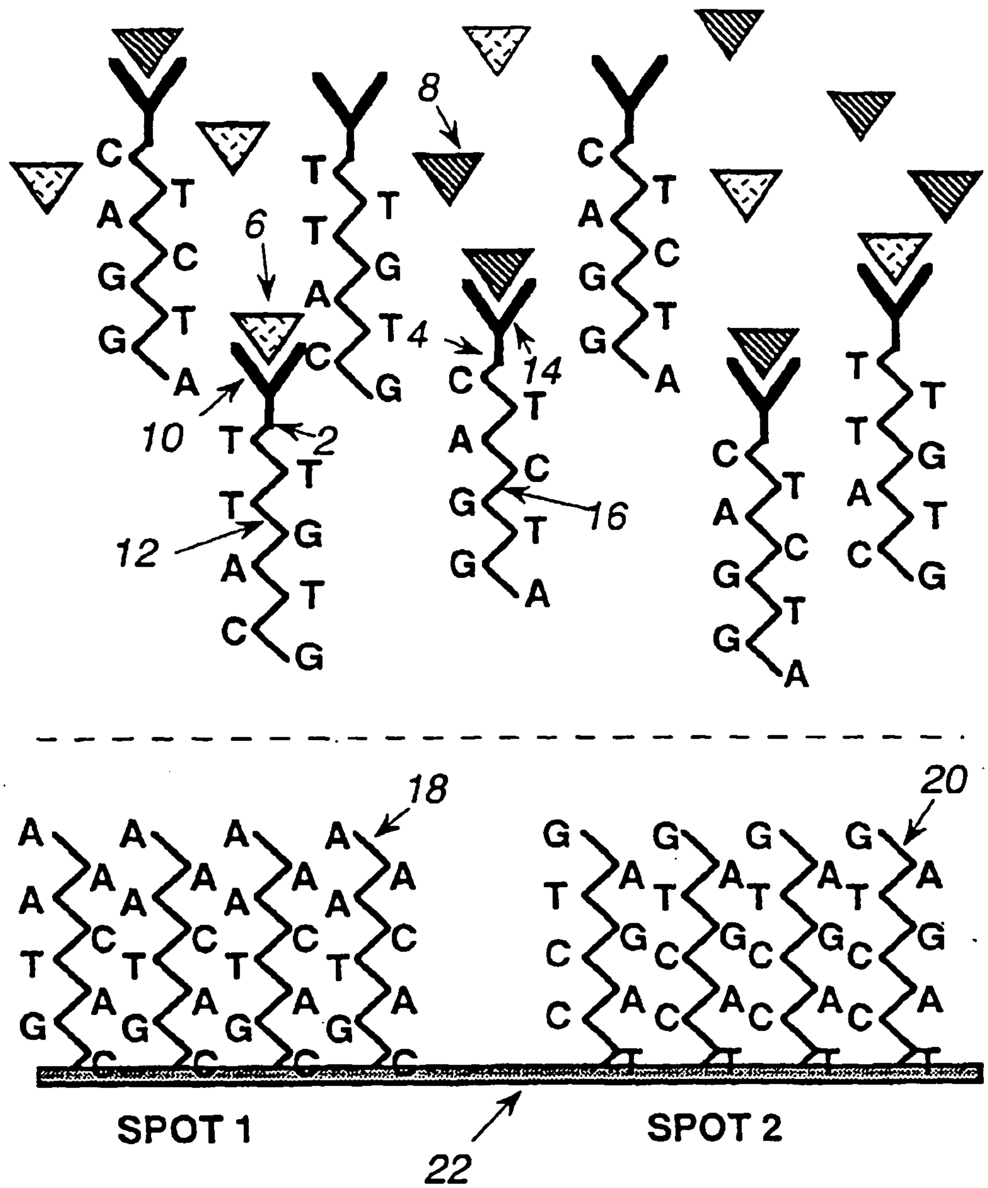

Figure 1

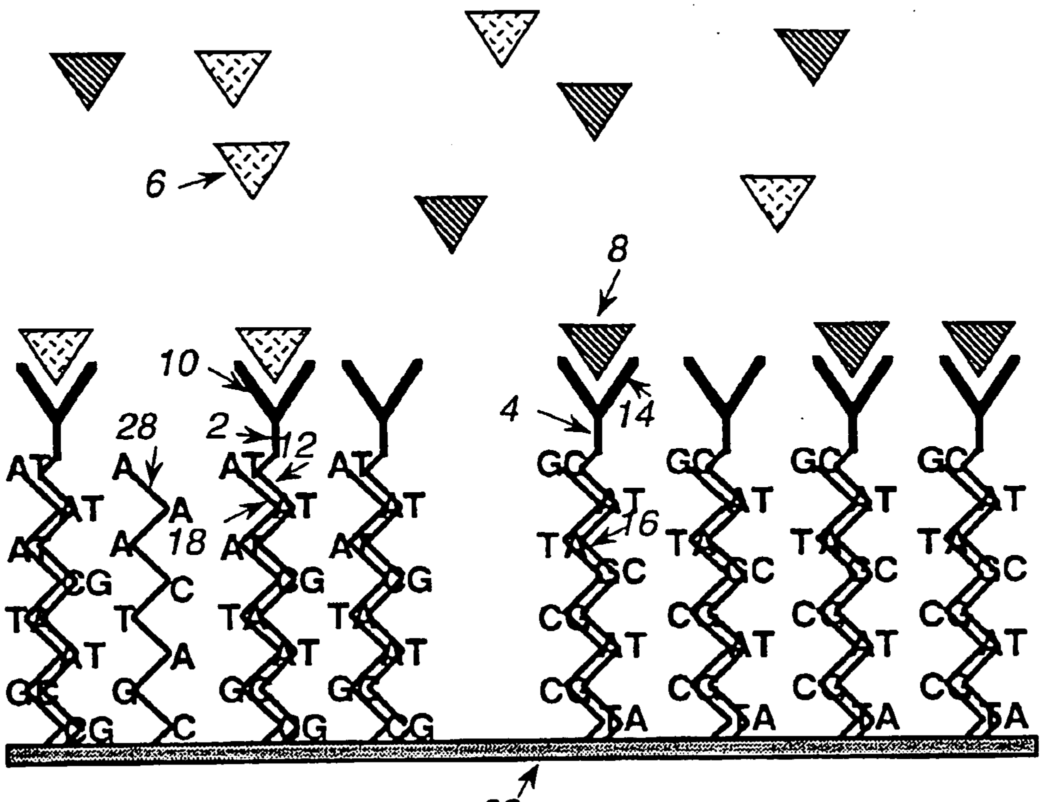

Figure 2

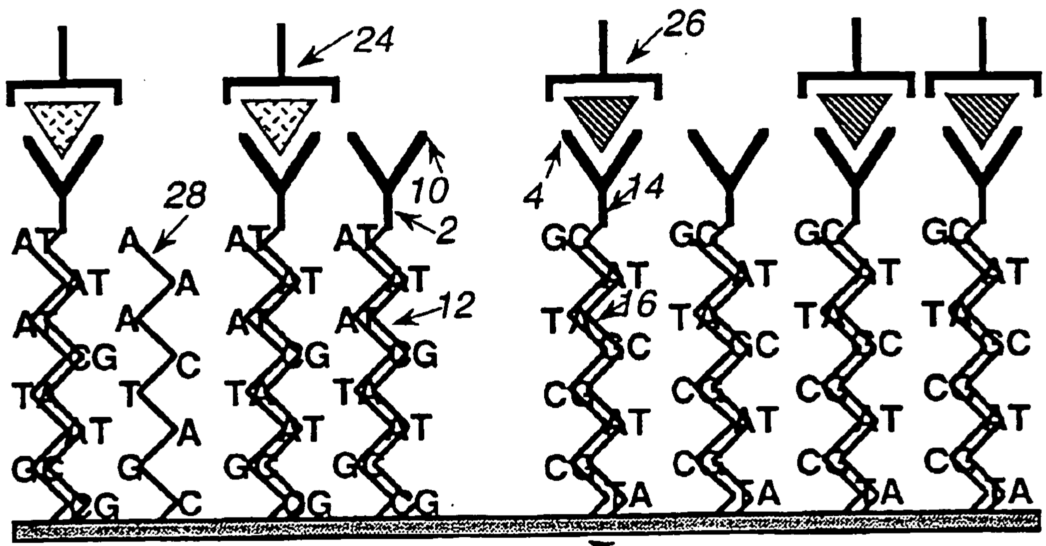

Figure 3

A Microspot Sandwich TSH Assay Using Solid-phased Anti-TSH Capture Antibody Tagged with Oligonucleotide

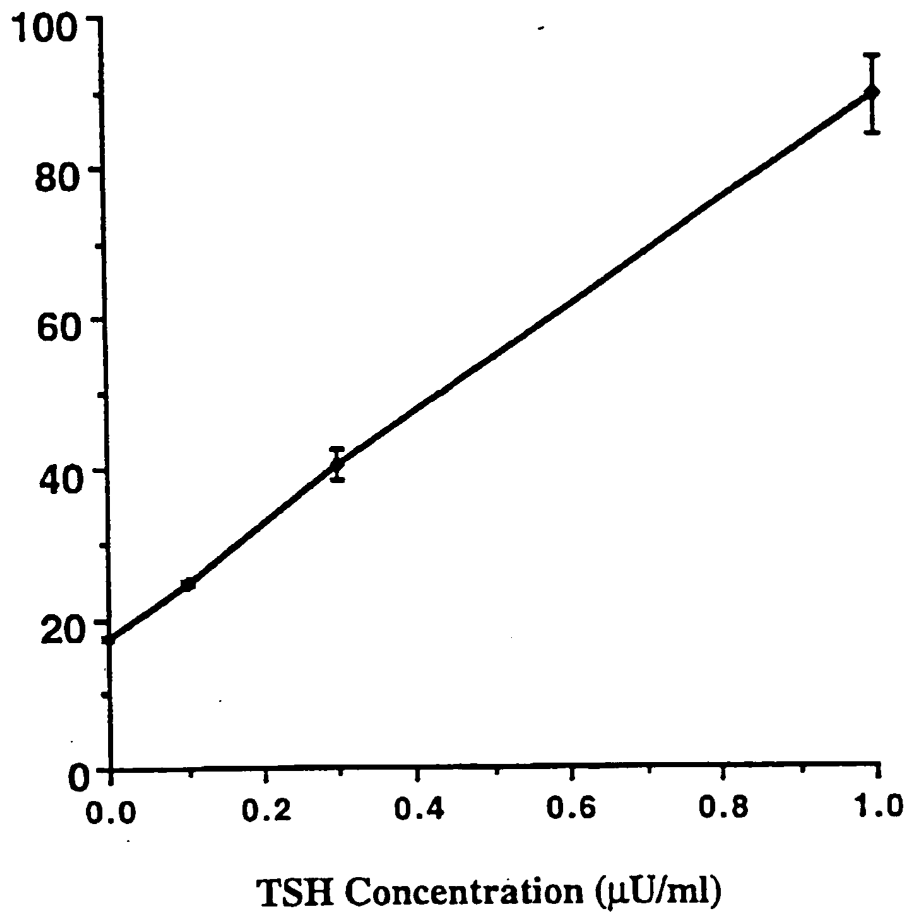

Figure 4